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”Pharmacokinetics and inner ear transport of cisplatin”

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In honour of my late grandmother and pride in my beloved children, Gustav and Ida.

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Enheten för öron- näs- och halssjukdomar

“Pharmacokinetics and inner ear transport of cisplatin”

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ABSTRACT

Background

Cisplatin is a commonly used platinum anti-cancer drug. Regrettably cisplatin has dose-limiting ototoxic side effects, e.g. the drug can induce an irreversible hearing loss. The ototoxic mechanisms of cisplatin have not been elucidated in the human ear and no clinically useful oto-protectors are yet available. Cisplatin is a necessary part of many treatment regimes. Its beneficial therapeutic effects might be reduced if cisplatin was excluded from the treatment in order to protect the hearing function. In this work the ototoxic effects of cisplatin are studied with the aim to better understand the mechanisms behind the irreversible hearing loss induced by this drug. Oxaliplatin is a second generation platinum-derivative anti-cancer drug, free from ototoxic side effects in clinical practice. The effects of oxaliplatin on the inner ear have been studied in this work and the results are compared with cisplatin treatment. The two drugs differ regarding both anti-cancer effects and side effects, which could be attributed to differences in pharmacokinetic factors, cellular uptake and apoptotic mechanisms. The thioredoxin redox system with the enzyme thioredoxin reductase (TrxR) was studied in cochleae due to a suggested DNA-independent apoptotic mechanism of the hair cells. The cochlear pharmacokinetics of cisplatin was assessed and the transport protein organic cation transporter 2 (OCT2) was studied in relation to the ototoxic effect of cisplatin.

Material and methods

Cultured human colon carcinoma cells and cell cultures of rat organ of Corti were used for apoptosis studies *in vitro* following exposure to cisplatin and oxaliplatin. Cisplatin and oxaliplatin were administered i.v. to guinea pigs, followed by *in vivo* sampling of blood, cerebrospinal fluid (CSF) and scala tympani (ST) perilymph. Liquid chromatography with post-column derivatization was used to determine the concentration of parent drug in the samples. Electrophysiological hearing thresholds and the loss of hair cells were assessed to evaluate their ototoxic effects. Phenformin, a potential blocker of OCT2 was administered and the ototoxic side effect of cisplatin was evaluated. For immunohistochemical studies, cochlea from rat, guinea pig and pig were used, where TrxR and OCT2 were evaluated in the cochlea. TrxR-assays were used to measure the TrxR activity in cochlear tissue, both *in vivo* and *in vitro*.

Results

The results from the *in vitro* studies showed that addition of either cisplatin or oxaliplatin to the culture medium in organ of Corti cell cultures caused a similar amount of outer hair cell loss and inhibition of TrxR activity. Cisplatin exposure to cultured human colon carcinoma cells also reduced the activity of TrxR. The results from the *in vivo* studies showed that a considerable concentration of cisplatin was present in ST perilymph as compared with weak concentrations of oxaliplatin after high dose oxaliplatin i.v. Ten minutes after cisplatin administration, its concentration in ST perilymph was 4-fold higher in the basal turn of the cochlea as compared to the apex. Cisplatin could be analysed in ST perilymph for up to 120 min. Phenformin i.v. did not reduce the ototoxic side-effect of cisplatin. Positive immunoreactivity to TrxR was evident in both hair cells and spiral ganglion cells. Futhermore, OCT2 was expressed in the supporting cells of organ of Corti and in the spiral ganglion cells.

Conclusion

The transport of cisplatin to the vulnerable cells of hearing seems to be of major importance for the ototoxic effects. An early high concentration of cisplatin in the base of the cochlea and delayed elimination of cisplatin from ST perilymph may be related to the cisplatin-induced loss of outer hair cells in the basal turn of the cochlea. Cisplatin and oxaliplatin both cause similar ototoxic effects when the organ of Corti is directly exposed *in vitro*. The thioredoxin redox system with the TrxR enzyme may well play a critical role in cisplatin-induced ototoxicity. The presence of OCT2 in the supporting cells indicates that this transport protein is primarily not involved in the uptake of cisplatin from the systemic circulation but rather from the deeper compartments of the cochlea. The knowledge elicited in this work will hopefully suggest objectives for further studies in order to develop oto-protective treatments to preserve the hearing of cisplatin treated patients.

LIST OF CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following original papers, referred to in the text by their roman numerals.

I. **Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity.**
Hellberg V, Wallin I, Eriksson S, Hernlund E, Jerremalm E, Berndtsson M, Eksborg S, Arnér ES, Shoshan M, Ehrsson H, Laurell G.
J Nat Cancer Inst 2009; 101(1):37-47

II. **Cochlear pharmacokinetics of cisplatin – an *in vivo* study in the guinea pig.**
Hellberg V, Wallin I, Ehrsson H, Laurell G.
Laryngoscope 2013; 123(12):3172-3177

III. **Cisplatin and oxaliplatin are toxic to cochlear outer hair cells and both target thioredoxin reductase in organ of Corti cultures.**
Dammeyer P, ***Hellberg V***, Wallin I, Laurell G, Shoshan M, Ehrsson H, Arner ES, Kirkegaard M.
Acta Otolaryngol 2014; 134(5):448-454

IV. **Immunohistochemical localization of OCT2 in the cochlea of various species.**
Hellberg V, Gahm C, Wei Liu, Ehrsson H, Helge Rask-Andersen, Laurell G.
Submitted to The Laryngoscope

ABBREVIATIONS

ABR	auditory brainstem response
AUC	area under the concentration-time curve
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
GSH	glutathione
IHC	inner hair cell
MHC	monohydrated complex of cisplatin
OCT2	organ cation transporter 2
OHC	outer hair cell
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SC	supporting cell
SGC	spiral ganglion cell
ST	scala tympani
SM	scala media
SV	scala vestibuli
Trx	thioredoxin
TrxR	thioredoxin reductase
UF	ultrafiltrate

1. INTRODUCTION

1.1 Ototoxicity

Ototoxicity in its widest sense comprises several drug-induced toxic effects on hearing and balance. Ototoxicity can be either irreversible or reversible. Loop diuretics such as furosemide and the anti-malaria drug quinine can induce a reversible ototoxic effect in the cochlea and/or the vestibular organ. Aminoglycoside antibiotics and the anti-cancer drug cisplatin can cause irreversible ototoxic effects. In the clinical setting, cisplatin-induced hearing loss is dose-dependent, with an obvious individual variability in susceptibility [1]. In Swedish patients cisplatin is the most common cause of drug-induced hearing loss which is often accompanied by tinnitus [2-4]. The cellular mechanisms underlying the ototoxic effects are complex and not fully elucidated. Although much experimental research has been performed, no clinical otoprotector is yet available that protects the hearing without counteracting the antineoplastic effects of cisplatin. In this work the ototoxic effects of cisplatin were studied with the intention to better understand the mechanisms causing the irreversible hearing loss induced by the drug.

1.2 Cancer

Cancer is an uncontrolled proliferation of a cell population. Treatment of cancer often utilizes multiple modalities such as surgery, radiation and chemotherapy, alone or in combinations.

1.3 Apoptosis

Apart from apoptosis of cells in healthy physiology, a pathological form of apoptosis is significant in diseases such as Parkinson's disease and ALS. Apoptosis must be distinguished from necrosis which is described to be a traumatic cell death that can be secondary to mechanical trauma, ischaemia, or thermal injury. This causes cells to swell, releasing cytoplasm and induce an inflammatory response. Apoptosis is a complex energy dependent process of programmed cell death [5]. First the cell forms cytoplasmic blebs and apoptotic bodies before it starts to shrink, resulting in condensed cytoplasm and fragmented nuclei. The cell membrane is often intact and there is no inflammation. Apoptosis follows two main ways pathways: the extrinsic or the intrinsic. The extrinsic signalling pathway initiates apoptosis via transmem-

brane death receptors that are members of the tumour necrosis factor receptor gene superfamily. Death receptors mediate apoptosis via an activation of the effector caspase 3. The intrinsic pathway is initiated through the cell mitochondria, inducing caspase 3 activation. Calcium is also important for the apoptotic pathways [6].

1.4 Chemotherapy

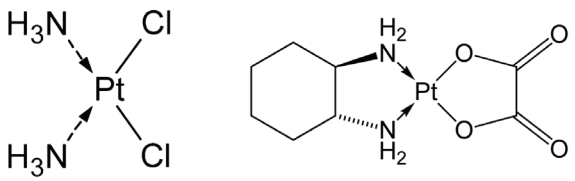
Chemotherapeutic drugs induces apoptosis primary in cancer cells having a rapid growth rate. The use of chemotherapy in reality requires a balance between killing cancer cells while ensuring a sufficient survival of healthy cells, in order to minimize side effects. Consequently anti-cancer drugs have both dose dependent and dose limiting side effects. The cytotoxic effects are mediated by various mechanisms. The drug can have a direct deoxyribonucleic acid (DNA) binding capacity, or it can inhibit the DNA-independent cellular machinery that affects division of cells. Anti-cancer drugs are divided into different categories, depending on differences in cytotoxic effect. Anti-metabolites such as methotrexate block enzymes that are necessary for DNA synthesis. Alkylating agents such as cyclophosphamide and the platinum-based drug, as cisplatin are cell-cycle-independent and their major anti-neoplastic effects are mediated by formation of DNA intrastrand cross-links.

Cisplatin has been used in cancer treatment for more than 40 years. It is often combined with other anti-cancer drugs. New platinum-based anti-cancer drugs have been developed with the purpose of limiting the side effects and to improve the anti-cancer effect of cisplatin. Several drugs have entered clinical trials but only carboplatin and oxaliplatin have been approved worldwide, but cisplatin is still the most potent of these drugs [7]. Carboplatin was introduced in the late 1980s and it is currently used to treat ovarian cancer and small-cell lung cancer [8]. The major dose-limiting side effect of carboplatin is myelosuppression [9]. Ototoxic and nephrotoxic side effects are mentioned as common, but are rarely seen in clinic. Oxaliplatin was studied in this work due to the fact that it does not affect the hearing and our experimental results are therefore of interest when comparing this drug with cisplatin.

1.5 Cisplatin

Cisplatin (cis-diammine-dichloro-platinum, with the chemical formula $H_6C_{l2}N_2Pt$, Platinol®) is a small, uncharged, water-soluble molecule with a molecular weight of 300 g/mol. The central platinum core is surrounded by two stable amine groups and two reactive chloride groups (Fig. 1). The platinum-based drug cisplatin was first discovered by the American chemist Barnett Rosenberg (1926 – 2009). In 1965 Rosenberg and his colleague showed that bacterial cell division could be inhibited by cisplatin [10]. Four years later they described the cytotoxic effect of cisplatin [11]. The first clinical study of cisplatin was presented in 1972 [12] and in 1978 cisplatin was approved for the treatment of testicular and ovarian cancers in the USA. Today cisplatin is used in the treatment of testicular cancer [13], ovarian cancer, bladder cancer, lung cancer and head and neck cancer [14] and against paediatric malignancies [15] such as medulloblastoma [16], osteosarcoma [17] and neuroblastoma [18]. The use of cisplatin against testicular cancer has substantially improved the survival rate.

In clinical use, cisplatin is usually administrated i.v. in a high-dose or a low-dose regimen at a dose of 40-75 mg/m² administer in repeated cycles. Cisplatin can also be administrated intraperitoneally [19]. About-one third of the patients treated with a high dose regimen (doses exceeding 60 mg/m²) develop a hearing loss [1, 3, 20]. When severe hearing loss occurs during cisplatin treatment the drug is often excluded and the carcinogenic effects of treatment may then be reduced [2, 4]. Besides its ototoxic side effect, cisplatin has nephrotoxic side effects. The renal damage caused by cisplatin is often successfully alleviated by pre-treatment with hydration and forced diuresis [21]. Cisplatin can also induce gastrointestinally toxic and cause neurological side effects giving rise to a distal sensory neuropathy [22, 23].



Cisplatin Oxaliplatin

Fig. 1. The molecular formulae of cisplatin and oxaliplatin.

1.6 Oxaliplatin

Oxaliplatin (oxalate (1*R*,2*R*-cyclohexandiamine) platina (II), with the molecular formula $C_8H_{14}N_2O_4Pt$, Eloxatin®) is a small uncharged molecule with a molecular weight of 397 g/mol, having a central platinum core and reactive ligands (Fig. 1). In oxaliplatin the two chloride ligands have been replaced by a single oxalate ligand, and is thus less resistant than cisplatin as the different adducts formed with DNA [24] prevent binding of DNA repair proteins. No ototoxic or nephrotoxic side effects have been reported [25]. The oxalate ligand also greatly alleviates the severity of the side effects of the drug compared with cisplatin [24]. Oxaliplatin was first used in the USA in 2002 [26]. It is prescribed to treat metastatic colorectal cancer [27], ovarian cancer [28], metastatic breast cancer [29], pancreatic cancer [30] and esophagogastric cancer [31]. In clinical practice oxaliplatin is i.v. administered in repeated cycles at doses of 85-130 mg/kg [32]. Oxaliplatin can also be administered locally as an intraperitoneal chemotherapy [33, 34]. Neurotoxicity is the dose-limiting effect of oxaliplatin [35-37].

1.7 Cytotoxic effects of cisplatin and oxaliplatin

Due to different toxic profiles both in targeting cancer and side effects, there might be a variation in the specific drug transport and/or apoptotic mechanisms for cisplatin and oxaliplatin. Platinum based drugs probably enter cells mainly through passive diffusion but active transport mechanisms have also been proposed [38-43]. Cisplatin [44, 45] and oxaliplatin [40, 46-48] both form cross-links between the DNA strands and thereby inhibit cell transcription and replication. At equimolar doses, oxaliplatin produces fewer DNA adducts than to cisplatin but is equally potent [49]. This could be explained by difference in platinum-DNA adducts and differences in the DNA repair mechanism [46, 50]. There are also DNA-independent mechanism of apoptosis [6] involved which has been demonstrated to be caused in enucleated cells by inducing endoplasmic reticulum (ER) stress and a caspase activation [51, 52]. It has been shown that cisplatin interacts with RNA, mitochondrial DNA [53-55] and proteins involved in the antioxidant systems, energy production and with cell signalling peptides [56-58]. Today it is known that cisplatin and oxaliplatin increases the cytoplasmic levels of toxic free radicals, i.e. reactive oxygen species (ROS), in the apoptotic process of the cell [34, 59, 60].

1.8 Redox system

Oxidation reactions are necessary for cellular life and cells have several oxidative substances and corresponding redox systems. The oxidation process produces ROS that can initiate reactions, leading to cell apoptosis. Oxidative stress is potentially dangerous and is involved in the development of many diseases including several forms of cancer [61]. A number of antioxidants such as glutathione and vitamins A, C and E are used for the treatment or prevention of diseases. The glutathione (GSH) and the thioredoxin (Trx) systems are the two major redox systems in cells [62].

The Trx-system contains the 12 kDa protein thioredoxin (Trx) and the enzyme thioredoxin reductase (TrxR). Trx has 2 cystine groups that can cycle between an active reduced dithiol form and an oxidized disulphide form. The reduced form of thioredoxin has strong anti-oxidative properties [63]. After the Trx protein is oxidized, the enzyme TrxR regenerate the oxidized Trx to an active, reduced form [64]. The Trx redox system has been shown to be involved in the cytotoxic effect of cisplatin and oxaliplatin [65-67]. Cisplatin and oxaliplatin are known to bind to the selenocysteine-containing active site on TrxR, a site that is important for the reduction capacity of TrxR. The activity of the enzyme is thereby reduced [65, 66]. The Trx redox system is thereby inhibited and the level of ROS is increased which may be deleterious for cells [67].

Due to the fact that hair cells are non-replication, a DNA-independent mechanisms of apoptosis are supposed to be involved and it is shown that cisplatin induces an oxidative stress pathway, i.e. reducing anti-oxidation enzymes in the cochlea [68, 69]. The concentration of ROS is thereby increased. This induces an oxidative stress that activates apoptotic mechanisms in the inner ear. The exact mechanisms are far from fully clarified. Intracellular antioxidants related to the GSH system are previous studied in relation to cisplatin induced apoptosis of the hair cells [56, 68, 70, 71]. In this work, the Trx redox system is studied in relation to the cytotoxic effect of cisplatin and oxaliplatin and for the involvement in cisplatin-induced ototoxicity. TrxR is studied immunohistohemically in the rat and guinea pig cochlea. After cisplatin and oxaliplatin treatment the activity of TrxR are measured both *in vitro* and *in vivo* i.e. in cultured colon carcinoma cells, in rat organ of Corti cell cultures and in cochlear tissue from guinea pigs.

1.9 Pharmacokinetics

The pharmacokinetics of a drug is studied by analysing the drug concentrations at different time points in different compartments, which can be summarized as what is happening to the drug in the body. In this work the pharmacokinetic profiles of cisplatin and oxaliplatin were assessed in blood, cerebrospinal fluid (CSF) and in the most voluminous cochlear fluid, the perilymph. "The area under the concentration time curve" (AUC), peak concentration and the elimination half-time of the drug were measured and calculated. In blood, cisplatin and oxaliplatin bind to albumin, whereby the drugs lose most of its cytotoxic properties [72, 73]. The unbound fraction of the parent drug represents the "active" part of the drug [72, 74]. Either cisplatin or oxaliplatin are enzymatic metabolized and both drugs are excreted via renal filtration. In this work all pharmacokinetic results derive from the concentration of the unbound, active part of cisplatin and oxaliplatin, respectively [75, 76]. In previous studies it has been shown that the blood elimination half-life of cisplatin was approx. 23 min after patients had undergone a 1-hour infusion of cisplatin 100 mg/m² [77, 78] and for oxaliplatin, 14 min when patients received 85 mg/m² oxaliplatin in 2-hour i.v. infusion [73, 75]. The pharmacokinetic profile of cisplatin in blood is of important for drug transport to the inner ear, as is shown in this work [79, 80].

1.10 The cochlea

Anatomy

Fig. 2 shows the anatomy of the cochlea. The human cochlea consists of two and a half turns (approx. 31 mm from the base to the apex) compared to three and a half turns in the guinea pig (approx.19 mm from base to apex) [81, 82]. The bony cochlea harbours the membranous labyrinth with three fluid-filled compartments i.e. scales; scala media (SM), scala tympani (ST) and scala vestibuli (SV). Reissner's membrane separates SM from SV and the basilar membrane separates SM from ST. Helicotrema is a passage in the most apical part of the cochlea, connecting ST with SV. There are two openings in the cochlear bone i.e. the oval and the round window. The stapes foot-plate from the middle ear bone is attached to the oval window that is heading to SV. The round window membrane is connected to ST. The modiolus is the central bony part of the cochlea containing the spiral ganglion cells (SGCs) and the peripheral neurons of the cochlear nerve located in Rosentahl´s canal. The or-

gan of Corti, with the inner hair cells (IHCs) and outer hair cells (OHCs) and surrounding supporting cells (SC) are situated on the surface of the basilar membrane.

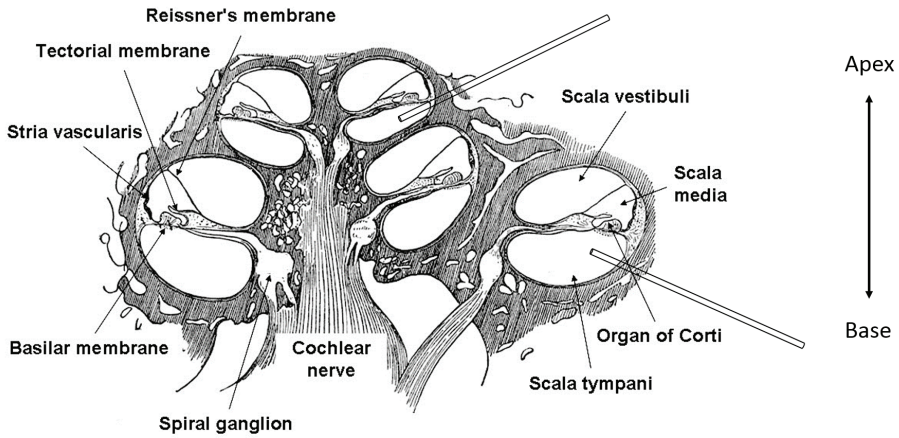


Fig. 2. A cross-section of the cochlea with its basal and apical parts. The capillary tubes show from where scala tympani perilymph was aspirated from both the cochlear base and apex. (Gray´s Anatomy, Wikimedia Commons)

Cochlear fluids

There are two different fluids within the cochlea. Endolymph has a high concentration of potassium and a low concentration of sodium, very much like the intracellular fluid. SM is filled with endolymph [83, 84]. Endolymph is believed to derive from stria vascularis; it regulates the endolymphatic potential [85]. Perilymph on the other hand has a high concentration of sodium and a low concentration of potassium. It is very much like the extracellular fluid. ST and SV are filled with perilymph i.e. ST perilymph and SV perilymph. Perilymph is believed to be produced "locally" and does not derive from CSF as supposed earlier. ST perilymph has an extremely slowly turnover rate and the circulation route of the fluid is from base to apex of the cochlea [86]. Cortilymph fluid was previus described to be the fluid localized around the basal parts of the hair cells, but today it is known that it is ST peilymph that is surrounding the OHCs and the tunnel of Corti [87]. The ionic composition of endolymph and perilymph does not differ significantly between humans and other species [82, 88-91]. The total volume of ST perilymph in a guinea pig is calculated to be around 4.7 µL and in a human cochlea, 29.2 µL [90,

91]. ST perilymph communicates with CSF via the cochlear aqueduct that is a passage situated very close to the round window in the base of the cochlea. This passage is very narrow and is known to be open in animals, but its morphology in the human cochlea is uncertain [92]. In this work, ST perilymph was sampled from the basal turn and from the apex of the cochlea, shown in Fig. 2.

Organ of Corti

Fig. 3 shows the organ of Corti with the IHCs, OHCs and the surrounding SCs. The pear-shaped IHCs are the primary sensory receptors that convert mechanical energy, i.e. movements in the basilar membrane, to nerve impulses with an afferent nerve transport to the central nerve system. The cylindrical OHCs are connected mainly to efferent neurons via the olivocochlear pathway, an efferent feedback system, which modulates hearing sensitivity [93-95]. The OHCs are also known as the cochlear amplifier [96]. They acts as "motors" that compensate for energy loss in the cochlea along "the travelling wave" [93, 97]. The cochlea has one row of IHCs and three rows of OHCs. The human cochlea has about 12,000 OHCs and 3,500 IHCs [98]; the guinea pig has approx. 6,500 OHCs and 1,900 IHCs [99].

Stereocilia are located on the apical surface (cuticular plate) of the hair cells. The tallest tips of the OHCs stereocilia are embedded in the overlying tectorial membrane, whereas the tips of the stereocilia on the IHCs are not. The human OHC has about 50-150 stereocilia, localized in three of four rows with a V-shaped configuration. The human IHC has about 60 stereocilia situated in more flat U shape, and they are shorter in the base of the cochlea. Stereocilia have cross-linked actin filaments from the tip to the base of the stereocilia [100]. The basal parts of the OHCs are connected to ST perilymph [101] while their most apical parts with the stereocilia are situated in the endolymph [100, 102].

The SCs are also terminally differentiated cells. They are not only important the bolstering of the hair cells, they also have an important function in the recycling of potassium that is released from the hair cells, thereby maintaining the action potential [85, 103]. Deiters´ cells connect the OHCs to the basal membrane [104, 105]. The inner sulcus cells [85, 106] are situated medially. The inner and outer pillar cells form the tunnel of Corti. Hensen´s cells and outer sulcus cells (Claudius´ cells) [107] are situated laterally toward stria vascularis.

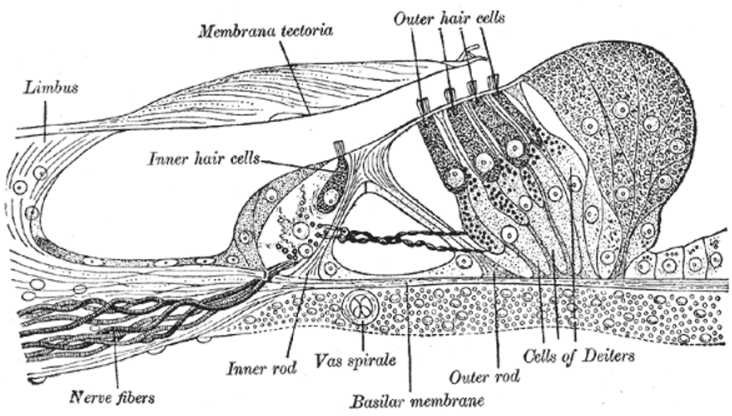


Fig. 3. Organ of Corti (Gray´s Anatomy, Wikimedia Commons)

Basal membrane

The basilar membrane forms the floor of SM. It consists of connective tissue and its anatomical structure is of a paramount importance for separating sound frequencies. It is wider in cochlear apex, generating low frequencies and in the base of the cochlea where it is more narrow and stiffer, generating high frequencies of sound [108].

Spiral ganglion cells

Spiral ganglion cells (SGC) are the cell bodies of the neurons in the cochlear nerve. The SGCs are located in the modiolus of the cochlea. The carry a peripheral neuron that is leading towards the organ of Corti and a process connected with the cochlear nucleus. There are two different types of SGC, types I and II. The myelinated type I cells (95%) are relatively large cells and have a pronounced nucleus, many mitochondria, Golgi apparatus, ribosomes, neurofilaments and an endoplasmic reticulum [95]. The peripheral neuron of type I cells is afferently attached to the IHCs [109]. The unmyelinated type II cells (5%) are rather small and the peripheral neuron is mainly efferently connected with the OHCs [95, 107].

Stria vascularis

Stria vascularis is the vascularized epithelium in the lateral wall of the cochlea. It has three layers of cells i.e. the marginal cells leading toward the endo-

lymph in SM, the intermediate cells, and the basal cells. By active processes stria vascularis maintains a high concentration of potassium in SM thereby giving a positive potential of +80 mV [85].

Spiral ligament

Stria vascularis and the more laterally situated spiral ligament are known as the lateral wall of the cochlea. The spiral ligament supports stria vascularis and consists mostly of fibroblast but also has a vascular epithelium and seems to be involved in the re-cycling process of potassium [106, 110].

1.11 The ear and hearing

The sense of hearing is an extremely complex process. Sound waves pass through the auditory canal where upon the tympanic membrane starts to vibrate. The middle ear with the ossicular chain i.e. the malleus, incus and stapes, transfer mechanical energy via movements in the oval window. This energy is further converted by the perilymph, creating a wave on the basilar membrane that travels from the base toward the cochlear apex i.e. the travelling wave. Movements in the basilar membrane deflect the stereocilia in the covering tectorial membrane.

The IHCs converts mechanical energy into nerve impulses in the central nervous system. This is due to opening of specific mechanically gated ion channels when the stereocila are deflected in the direction of the tallest stereocilia. This induces an influx of and potassium from the endolymph enters the IHCs which are then depolarized. Calcium channels open resulting in release of neurotransmitters (i.e. glutamate), generating nerve impulses in the attached afferent fibres in the base of IHC. Nerve impulses are transmitted via the afferent fibres of the type I SGCs and further via the cochlear nerve (the cranial nerve no.VIII, vestibulocochlear nerve) to the cochlear nucleus in the brain stem and then processed to the auditory cortex in the temporal lobe. The auditory sensory epithelium is organized along specific frequencies distributed along a tonotopic axis along the cochlea to a tonotopic map in the auditory cortex [111]. When sound waves decreases, the sterereocilia are defleced toward the shortest stereocilia, the ion channels close. The function of the OHCs as a cochlear amplifier are of a paramount importance for the afferent nerve transport of the IHCs [93-96]. Disruption of the OHCs causes hearing loss at the associated frequencies.

1.12 Ototoxicity of cisplatin

Damage to hair cells that have a very low DNA-turnover leads to permanent hearing loss, i.e. an irreversible sensorineural hearing loss. Cisplatin treatment affects mostly the OHCs in the basal part of the cochlea, inducing hearing loss in high-frequencies. Hearing loss in the speech frequencies can also be impaired after high dose cisplatin treatment. In humans, the hearing loss becomes evident after some days after cisplatin treatment [105, 112]. Fig. 4 show a tone audiogram from a child treated with cisplatin.

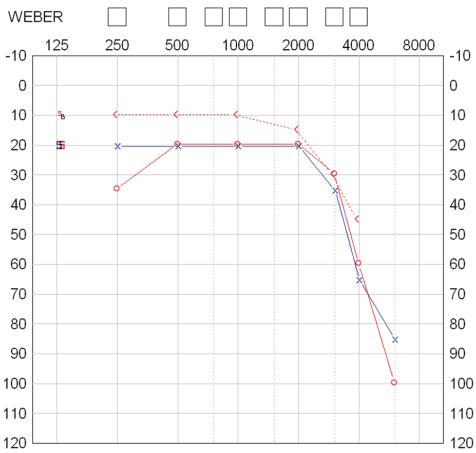


Fig. 4. A tone audiogram from a 4 years old child treated with cisplatin, showing a profound bilateral sensorineural hearing loss at the high frequencies of hearing. (Patient met at clinic.)

In animal studies the ototoxic effect is often determined with auditory brainstem response (ABR) and, histologically, loss of hair cells is calculated post mortem. When guinea pigs are given an ototoxic dose of cisplatin, a significant reduction in electrophysiological hearing thresholds, mostly at high frequencies, becomes evident after 96 hours. This is caused mainly by a damaged OHCs and SC in the base of the cochlea [105, 113-115]. Some studies have also reported lesions to the IHCs, SGCs [116-118] and stria vascularis [105, 113, 119, 120]. After high-dose treatment with cisplatin the morphological damage progresses towards the apical part of the cochlea [105, 114, 115] The OHCs in the first row are more severely affected than the second and third rows [105, 115]. In this work the ototoxic effect of cisplatin was evaluated in experimental animals by measuring the electrophysiological hearing thresholds with ABR and morphological studies were performed to calculate loss of the hair cells.

The individual variability in susceptibility of the ototoxic side effect of cisplatin is seen both in clinic and in animal studies [1, 121, 122]. Elderly patients [1, 123] and children [124] seem to be more susceptible to the ototoxic effect of cisplatin. When cisplatin is given as single high [1] or a high cumulative dose [1, 124] the ototoxic effect is more pronounced. Ekborn and co-workers (2000) showed that the severity of hearing loss due to cisplatin was not related to the drug infusion rate [125].

1.13 Nephrotoxicity of cisplatin

Cisplatin affects the endothelial cells in proximal tubules in the kidney [126]. The proximal tubule is responsible for active reabsorption of sodium chloride. Renal failure with reduced renal clearance produces elevated concentrations of S-creatinine and S-urea [127]. Renal damage due to cisplatin treatment can be alleviated by hydration and a forced diuresis [128]. Cisplatin-induced apoptosis of the renal cells is associated with oxidative stress pathways. Both GSH redox system [57] and Trx redox system appears to be involved in the nephrotoxic effect of cisplatin [129, 130].

1.14 Monohydrated complex of cisplatin

A major biotransformation product of cisplatin is called the monohydrated complex (MHC). MHC is formed when cisplatin is hydrolysed in water, thereby losing a chloride ion and gaining a water molecule [76] (Fig. 5). Environmental factors such as low chloride concentration and low pH favour the formation of MHC [74, 131]. MHC appears to be more toxic than to cisplatin [79, 132]. The positively charged MHC has a stronger affinity for the negatively charged DNA and this could be one explanation for the increased toxicity of MHC compared to cisplatin [45]. It is known that MHC generates ROS and the oxidative stress pathway might be involved in the nephrotoxic side effect of cisplatin [133, 134]. The relationship of MCH to the ototoxic side effect of cisplatin has not been fully evaluated, but MHC in ST perilymph was analyzed in this work

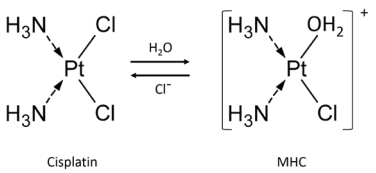


Fig. 5. The monohydrated complex (MHC) of cisplatin. Cisplatin is hydrolyzed in water; loses a chloride ion and gains a water molecule.

1.15 Inner ear pharmacokinetics of cisplatin

The knowledge of the distribution of and elimination of cisplatin from the inner ear is limited. For pharmacokinetic studies, reliable *in vivo* models are needed as this type of knowledge cannot be obtained in human beings. In animal studies cisplatin can be administered systemically as a i.p. or i.v. infusion but it can also be instilled directly to the middle ear [135] or directly into the inner ear [136, 137]. Laurell and co-workers (1995), showed that a paramount concentration of cisplatin could be measured in ST perilymph after i.v administration of high-dose cisplatin [80]. It is know that the longitudinal flow of perilymph is a very slow from the base to the apex of the cochlea [138] and cisplatin seems to be transported by longitudinal diffusion. There is also diffusion between different compartments of the cochlea, followed by a elimination through the vascularized tissue [139, 140]. It is also known that the concentration of cisplatin is greater in perilymph compared to the endolymph, following systemic administration [141]. In this work, the pharmacokinetic distributions of cisplatin were studied in the ear after i.v. bolus dose of cisplatin to guinea pigs. The concentration of cisplatin in blood and its transport to the inner ear is of basic is importance for understanding of the inner ear pharmacokinetics of cisplatin, as was also found in the present work.

1.16 Inner ear barriers

Cisplatin was initially thought to be transported to different compartments in the body by passive diffusion [142]. It is not known how cisplatin enters the perilymphatic and endolymphatic compartments in the inner ear, but passive diffusion and an active transport using specific transport proteins have earlier been discussed [39, 143]. Two barrier systems are described, partly with the function of protecting the inner ear from toxic substances: the intrastrial fluid–blood barrier and the blood–perilymph barrier. The vascular supply to the cochlea is of a paramount importance for these two barriers.

The main arterial vessel to the cochlea derivates from the basilar artery, followed by the anterior inferior cerebellar artery, which gives off a branch to the labyrinthine artery (inner ear artery), a terminal artery without collaterals. The common cochlear artery ends up in the spiral modiolar artery. The spiral modular artery give branches of radiating arterioles. One branch terminate in capillaries of stria vascularis and the spiral ligament [144, 145]. Another branch terminate in capillaries in spiral lamina and in the basal membrane in location under the tunnel of Corti.

The intrastrial fluid–blood barrier

The concept of a blood–labyrinth barrier has been used previously [146, 147], but the current definition of an intrastrial fluid–blood barrier is more common thanks to better knowledge of the structure and function of the barrier [148]. The capillary endothelium connected with tight junctions in stria vascularis and spiral ligament is representative of the intrastrial fluid–blood barrier [86, 140, 146, 147, 149-155]. Cochlear pericytes and perivascular resistant macrophage-type melanocytes are recently identified cells in the intrastrial fluid–blood barrier [148].

The blood–perilymph barrier

The vessels in the spiral lamina, and capillaries of the basal membrane are vessels that support the blood–perilymph barrier, but its structure is not precisely known [156].

1.17 Blood–CSF barrier

The choroid plexus in the ventricular system of the brain produces CSF and also acts as a barrier preventing toxic substances from passing into the CSF, i.e. a blood–CSF barrier [157]. The blood–CSF barrier is separated from the blood–brain barrier [158, 159]. CSF is produced when plasma is filtered from the blood through the epithelial cells into the choroid plexus which is sealed with tight junctions to prevent toxic substances from passing into CSF. Cisplatin can be measured in CSF [80, 160, 161] which means that the drug is able to penetrate the blood–CSF barrier *in vivo*. It has also been shown that cisplatin can be measured in human CSF [161-164]. The hydrophilic molecule cisplatin has a restricted access to the brain and is not primarily used for malignancy in the central nervous system. In combination with other anti-cancer drugs cisplatin is used to treat paediatric medulloblastoma [165].

1.18 Transport proteins within the cochlea

Various active mechanisms have been discussed regarding the transport of cisplatin to cells vulnerable for hearing loss. Whether or not there is a different transport mechanism involved in the influx of cisplatin from blood to the inner ear fluids, or for the uptake of cisplatin to OHCs, remains to be ascertained. One hypothesis in this work is that there is a drug-specific transport mechanism for cisplatin to the inner ear. The membrane protein organic cation transporter 2 (OCT2), and the copper transporter 1 (Ctrl) are the two most studied transport proteins regarding cisplatin toxicity. Various isoforms of OCTs have specific species- and tissue-distribution patterns [166, 167]. It was earlier demonstrated that both Ctr1 [38, 168] and OCT2 [39, 168] are actually involved in cisplatin-induced ototoxicity. In this work OCT2 has been studied regarding the ototoxic side effect of cisplatin [169].

1.19 Oto-protection

Although other researchers have attempted to contribute knowledge regarding protection of hearing in addition to cisplatin treatment, no oto-protective measures have actually succeeded in clinical practice. Knowing that cisplatin does increase the level of ROS in hair cells, several antioxidant therapies have been studied to offer oto-protection [170-172]. A number of sulphur-containing compounds, such as N-acetyl-cysteine, sodium thiosulphate [173] and D-methionine [174], are antioxidants with a high affinity to platinum species. Unfortunately these drugs also counteract the anti-tumour effects when they administered systemically [121]. Local application of antioxidants to the ear is therefor to be preferred [175-177] [177]. Another way for oto-protection is to block the uptake of cisplatin in the inner ear so as to prevent high cisplatin concentrations from reaching vulnerable auditory cells. When transport inhibitors and competitors such as copper sulphate [38] and cimetidine [39, 178, 179] are administrated i.v. they interact with active transport of cisplatin [180]. In this work, a potential blocker of OCT2 (phenformin) was studied as regards to the ototoxic sides effect of cisplatin.

2. AIMS

The overriding aim of the research presented in this thesis was cisplatin-induced ototoxicity and how it is affected by the pharmacokinetics of the drug, in particular as related to transport mechanism to and potentially protective systems in the inner ear. The specific aims of the studies, presented in the individual papers were:

Paper I

1. To study the cochlear pharmacokinetics of cisplatin and oxaliplatin, and ascertain if the pharmacokinetic distribution of the parent drug can explain the ototoxic side effects of cisplatin.
2. Study DNA-independent apoptotic mechanisms and suggested involvement of the Trx redox system in cultured colon carcinoma cells following treatment with cisplatin or oxaliplatin.
3. Evaluate if TrxR could be identified immunohistochemically in the cochlea.

Paper II

1. To study the distribution of cisplatin in ST perilymph, blood, and CSF after i.v. infusion.
2. To compare the concentrations of cisplatin in the base and apex of the cochlea.
3. Establish how long cisplatin could be measured in ST perilymph.

Paper III

1. To study the loss of hair cells following direct administration of cisplatin or oxaliplatin to cultured organ of Corti.
2. Study the Trx redox system in cochlear tissue after exposure to oxaliplatin or cisplatin, both in *vitro in* and *in vivo*.

Paper IV

1. To identify the cellular distribution of OCT2 in the cochlea in various species, using immunohistochemical methods.
2. Investigate if phenformin, a potential OCT2-blocker, could prevent cisplatin-induced ototoxicity *in vivo*.

3. MATERIAL AND METHODS

Experimental design, papers I-IV

Paper I

In vitro studies: Cultured colon carcinoma cells were used to study DNA-independent mechanisms of apoptosis and the activity of TrxR was assessed after treatment with cisplatin or oxaliplatin.
In vivo studies: Guinea pigs were given a high dose of i.v. oxaliplatin (16.6 mg/kg) or a low non-ototoxic dose of i.v. cisplatin (5 mg/kg). The pharmacokinetic distribution of the drugs was determined in blood, CSF, and ST perilymph up to 90 min after the parent drug was administered. Electrophysiological hearing thresholds were determined, using ABR, hair cells were counted and the platinum content was determined in cochlear tissue.
Morphological studies: The expression of TrxR was evaluated immunohistologically in guinea pig cochlea.

Paper II

In vivo studies: The pharmacokinetics of cisplatin in guinea pig cochlea was evaluated after i.v. administration of an ototoxic dose of cisplatin (8 mg/kg). Blood, CSF and ST perilymph were aspirated 10-180 min after drug administration and the concentration of cisplatin was assessed. ST perilymph was aspirated from both the cochlear base and apex during the first 30 min after drug administration.

Paper III

In vitro studies: The apoptotic effects on the hair cells and the activity of TrxR were evaluated in organ of Corti cell cultures exposed to either an equimolar dose of cisplatin or oxaliplatin.
In vivo studies: An ototoxic dose of cisplatin (8 mg/kg) or an equimolar dose of oxaliplatin (10.6 mg/kg) was administered i.v. to guinea pigs. After 24 hours, inner ear tissues were analysed for TrxR activity.

Paper IV

Morphological studies: The localization of OCT2 was characterized immunohistochemically in the cochlea of three different animal species.

In vivo studies: Phenformin, a potential OCT2-antagonist, was administered i.v. 30 min prior to an ototoxic dose of cisplatin (8 mg/kg) and a possible reduction of the ototoxic side effect of cisplatin was then evaluated.

3.1 Laboratory animals (I-IV)

Sprague-Dawley rats (Scanbur AB, Sweden) were used for immunostaining studies in papers III and IV. In paper III, cultures of organs of Corti were isolated from rat pups. Duncan-Hartley guinea pigs (BioJet, Uppsala, Sweden and Lidköpings Kaninfarm HB, Lidköping, Sweden) were used in all *in vivo* experiments in papers I-IV and for the immunostaining studies in papers I and IV. The animals were housed at the animal department of Karolinska University Hospital, Solna. All experimental animal studies in this thesis were performed in accordance with the ethical standards at Karolinska Institutet and Stockholms Norra Djurförsöksetiska Nämnd, with ethical approval N423/04, N213/05 (paper I), N 372/08 (paper II), N32/07, N372/08, N320/09 (paper III) and N135/11 (paper IV). In paper IV, cochlea from one pig, (*Sus scrofa*) was used in the immunostaining experiment. The pig cochlea was harvested from the Department of Anesthesia, Uppsala University Hospital, and in accordance with the ethical standards at Uppsala University and Djurförsöksetiska Nämnd, with ethical approval C315/5 (paper IV).

3.2 Drugs (I-IV)

Cisplatin (I-IV)

In the *in vivo* studies (I-IV) and in the *in vitro* studies (I), cisplatin (Platinol® 1 mg/ml, Bristol-Myers Squibb Pharmaceuticals, New York, USA) was used. In the *in vitro* studies (III), cisplatin (Cisplatin Mayne® 1 mg/ml, Hospira, LakeForest, USA) was administered.

Oxaliplatin (I and III)

For the *in vivo* studies (I and III) and in the *in vitro* studies (III) oxaliplatin (Eloxatin® 5 mg/ml, Sanofi-Aventis, Paris, France) was administered. For the *in vitro* studies (I) oxaliplatin (Oxaliplatin 5 mg/ml, Sigma-Aldrich Chemicals, Steinheim, Germany) was used.

Phenformin (IV)

For the *in vivo* studies, phenformin (phenformin hydrochloride 7.5 mg/ml, Sigma-Aldrich, Saint Louis, Mo, USA) was administered.

3.3 Surgical procedures *in vivo* (I-IV)

In general the guinea pigs were anaesthetized with ketamine (Ketalar®) 40 mg/kg and xylazine (Rompun®) 10 mg/kg i.m. for the electrophysiological hearing tests and for surgery. In paper II a combination of fentanyl (Hypnorm®) and fluanisone (Midazolam®) were administered i.m. for an adequate depth of anaesthesia. Atropine was given s.c. to reduce mucus in the airways. Lidocaine (Xylocaine®) was administered s.c. as local anaesthesia. During surgery the animals were placed on a heating pad and all surgical procedures were performed under clean conditions. The weight of each animal was checked daily and cisplatin or oxaliplatin treated animals were given a daily s.c. injection of 5 ml body warm saline. After the experiment the animals were deeply anaesthetized and euthanized with an overdose of sodium pentobarbital.

Catheter in jugular vein (I-IV)

After a skin incision in the anterior neck, the internal jugular vein was identified and catheterized. One catheter was used to deliver the drug and the other or to obtain blood samples [121, 125].

Dorsolateral surgical approach for access to the cochlear base (I and II)

The animal was lying on its belly, attached to a head holder. The bulla bone behind the ear was resected and the basal turn of the cochlea was exposed. The thin layer of mucosa on the bone was gently removed. A small hole was drilled in the basal turn of the cochlea for sampling ST perilymph [80, 181].

Ventrolateral surgical approach for access to the cochlear apex (II)

The animal was laid on its back, attached to a head holder. A transversal skin incision was made anteriorly in the neck and the animal was supplied with a tracheostomy to ease its ventilation. After blunt dissection medial to the man-

dible, the digastric muscle was reduced. The bulla bone was opened, thereby exposing the apical part of the cochlea.

Construction of a silicone cup (II)

For aspirating clear ST perilymph from the cochlear apex, a silicone cup was constructed [182] (Fig. 6). First the mucosa was removed from the apex bone and then a thin layer of glue was applied with a tiny stick. Several layers of silicone were applied to construct a "cup" over the cochlear apex. A sharp picker was used to make a small fenestra in the cochlear bone, and clear ST perilymph from the apex then slowly filled up the cup.

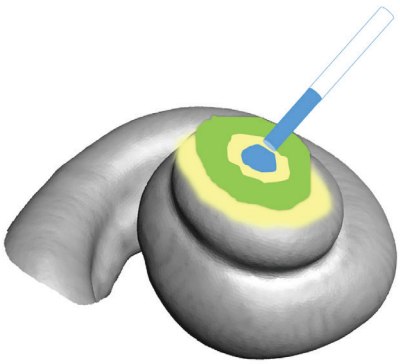


Fig. 6. Construction of the silicone cup on the cochlear apex.

Access to CSF (I and II)

In paper I the occipital bone was opened and dura mater was exposed. A small hole was made in the dura mater to get access to CSF from the subarachnoid space [80]. In paper II the skin was removed from the neck to prepare for a suboccipital approach to aspirate CSF from cisterna magna [183].

3.4 Sampling procedures *in vivo* (I and II)

Sampling of blood (I and II)

Blood was sampled by catheter from the internal jugular vein (opposite side from drug administration). To avoid blood clots, sodium heparin in sodium chloride was administered in each catheter. One blood sample (about 0.35 ml) was replaced with the same volume of sodium chloride.

Sampling of ST perilymph from the base of the cochlea (I and II)

In paper I a syringe was attached to a micromanipulator and the syringe tip was inserted into the drilled hole in the basal turn of the cochlea. One µl of clear ST perilymph was aspirated from the basal turn of the cochlea [80, 181]. In paper II capillary tubes were used to collect ST perilymph. One capillary was used to aspirate 1 µl clear ST perilymph from the basal turn of the cochlea [182].

Sampling of ST perilymph from "the cup", i.e. from the apex of the cochlea (II)

Capillary tubes were used for sequential sampling (no. 1–10) of 1 µl ST perilymph from the "silicone cup" on the cochlear apex [182]. (Fig. 6)

Sampling of CSF (I and II)

In paper I a syringe attached to a micromanipulator was used to aspirate clear CSF from the subarachnoid space [80]. In paper II a sharp syringe was used for a suboccipital puncture, and CSF was aspirated from cistern magna [183].

3.5 Drug analysis (I and II)

Samples of blood, CSF and ST perilymph were immediately stored on dry ice. Blood samples were ultrafiltrated and all samples were stored at –80ºC until analysis. Liquid chromatography (LC) with post column derivatization with *N,N*-diethyldithiocarbamate in a microwave field was used to determine the concentration of cisplatin, oxaliplatin, and MHC [75, 176, 184]. This is a unique method that analyses the parent drug not bound to proteins and thereby correlates to the true level of the active drug.

3.6 Auditory brainstem response (I, III and IV)

ABR was used to evaluate the electrophysiological hearing thresholds of the guinea pigs. The animals were anaesthetized and placed in a sound-proof box during the test [79, 185]. ABR threshold shifts were calculated as the difference between hearing thresholds obtained before vs. after drug treatment, expressed in dB [125]. Sound stimuli at different frequencies and sound levels were exposed direct to animal's external auditory canal. During ABR the

impulses from the hair cells to the auditory cortex are registered via electrodes inserted subcutaneously on the scalp. Various signal waves are recorded, corresponding to different neuroanatomical regions of the nerve impulse (waves I-V). Electrophysiological hearing threshold was defined as the lowest dB where a reproducible response could be recorded, adjusted in 5 dB steps. In this work the hearing thresholds were measured at the frequencies 6.3, 12.5, and 20 kHz.

3.7 Renal function (I)

Renal damage increases the concentration of creatinine and urea in blood. Blood samples from the guinea pigs were centrifuged and the serum promptly frozen. S-creatinine and S-urea were analysed with a spectrophotometric method at Karolinska University Hospital.

3.8 Surface preparations (I, III and IV)

Apoptotic studies and analyses of TrxR activity in organs of Corti cell cultures (III)

Organs of Corti (organ of Corti and spiral limbus) from rat cochlea of 2 or 3 day-old rat pupwere dissected in phosphate-buffered saline (PBS) and cultured in wells.

Analysis *in vivo* of TrxR activity in cochlear tissue (III)

After sacrificing each guinea pig, both temporal bones were immediately removed and the organ of Corti and lateral walls were isolated from the cochlea. Tissues from both left and right cochleae from the same animal were pooled in lysis buffer. Samples were promptly frozen on dry ice and stored at –80°C.

Counting loss of hair cells (I, III and IV)

The cochleae were prepared for morphological analysis of hair cell loss. After decapitation, the guinea pig cochlea was immediately removed from the temporal bone. The perilymphatic space was perfused and fixed with paraformaldehyde. The basilar membrane with the organ of Corti was exposed by removing the bone and surrounding tissue, and the remaining cells were labelled with phalloidin to visualize actin in the stereocilia of the hair cells.

Analysis of platinum in cochlear and renal tissues (I and IV)

Guinea pig cochleae were immediately removed from the temporal bones. The entire basilar membrane, including the organ of Corti and stria vascularis, was dissected and dried for analysis of platinum in tissue. In paper I, one kidney was also subjected to analysis.

Immunohistochemistry (I, III and IV)

For immunohistochemical studies, rat and guinea pig cochleae were immediately removed from the temporal bone after sacrifice. The cochleae were perfused and fixed in paraformaldehyde, then decalcified and embedded in paraffin. In paper IV, one kidney from each animal was removed and one half of the kidney was fixated in paraformaldehyde and then embedded in paraffin. The paraffin embedded cochlea and kidney tissue were sectioned 5 μm thick and mounted on microscopy slides. In paper IV the pig cochleae were prepared for cryosectioning, (embedded in Tissue-Tek (OCT Polysciences), rapidly frozen and sectioned at 8-10 μm) [186]. The frozen sections stored below –70°C prior to immunohistochemistry.

3.9 Counting loss of hair cells (I, III and IV)

Missing inner and outer hair cells were counted "blinded" in a fluorescence microscope from apex to base of the cochlea. The criterion for hair cell loss was scar formation. The results were presented in cochleograms, plotting the percentage of missing hair cells.

3.10 Platinum in cochlear and renal tissues (I and IV)

Platinum concentrations in cochlear tissue were determined by inductively coupled plasma mass spectrometry (ICP-MS by Analytica AB, now called ALS Scandinavia AB, Luleå, Sweden).

3.11 Immunohistochemistry (I, III and IV)

The paraffin sections of cochlea and kidney tissue were deparaffinized and the slides boiled for 10 min in an antigen unmasking solution (Vector Laboratories, Burlingame, Cal., USA) to improve the binding of the antibody to its epitope. All sections were incubated with blocking serum (5% goat serum) for 30 min at room temperature to minimize background staining. The prima-

ry antibody was excluded in the negative controls. The fluorescence signals were analysed in a light microscope (Zeiss axio scope; Carl Zeiss, Germany) and with a laser confocal microscope (Nikon TE2000, Nikon, Japan) [187].

TrxR (I and III)

In paper I, TrxR expression was investigated immunohistochemically in untreated guinea pig cochleae. Rabbit anti-rat thioredoxin reductase 1 (TrxR1) polyclonal antibodies, diluted 1:100 and 1:1000 (Upstate, New York, USA) were used as primary antibodies. All sections were incubated overnight at 4°C. Bound antibodies were visualized with indocarbocyanine, Cy3, (red colour) conjugated goat anti-rabbit antibody, diluted 1:1000 (Jackson Immuno Research Laboratories Inc, WestGrove, Pa). The secondary antibodies were applied for 60 min at room temperature.
In paper III, TrxR expression was investigated in cochleae from untreated rat pup (P2-P3). Rabbit anti-rat thioredoxin reductase 1 (TrxR1) polyclonal antibodies, diluted 1:1000 (Upstate Technology Laboratories, New York) were used as primary antibodies. Incubation with secondary antibody goat anti-rabbit (VectaStain Elite ABC kit, Vector Laboratories, Burlingame) for 40 min was followed by incubation with the ABC reagent and DAB-hydrogen peroxide solution according to VectaStain instructions.

OCT2 (IV)

In paper IV, OCT2 expression was investigated in cochleae from untreated rat, guinea pig and pig cochleae. Rat and guinea pig kidney was used as a positive control for OCT2 as reported in previous studies [188, 189]. Rabbit anti-rat organic cation transporter 2 (OCT2) polyclonal antibodies, diluted 1:100 (Alpha Diagnostic International, San Antonio, Tex., USA) were used as primary antibodies. All sections were incubated overnight at 4°C. OCT2 antibodies were visualized by means of indocarbocyanine, Cy3 (red colour) conjugated goat anti-rabbit antibody, diluted 1:200 (Jackson Immuno Research Laboratories Inc, West Grove, Pa). The secondary antibodies were applied for 60 min at room temperature.

Parvalbumin (IV)

In paper IV, double staining with parvalbumin was performed to identify the hair cells [187, 190]. Guinea pig and pig cochleae were used. Mouse anti-parvalbumin, diluted 1:250 (Chemicon International, Hants, England) was

used as primary antibodies. All sections were incubated overnight at 4°C. Parvalbumin antibodies were visualized by means of Alexa fluor 488 (green colour) conjugated goat anti-mouse antibody, diluted 1:400 (Invitrogen, Carlsbad, Cal., USA). The secondary antibodies were applied for 60 min at room temperature.

DAPI (I and IV)

The cell nuclei were counterstained with DAPI 4′,6-diamidino-2-phenylindole (blue colour). In paper I, DAPI (diluted 1:10 000) were administered for 1 min and sections were then mounted. In paper IV all slides were mounted with Vecta Shield mounting medium with DAPI, (Vector Laboratories) and nuclei from all sections were then counterstained.

3.12 Cell culture experiments with human colon carcinoma cells (I)

HCT116 human colon carcinoma cells were used in the cell culture experiment. Twenty four hours after incubation with cisplatin or oxaliplatin they were analysed by flow cytometry for apoptosis with antibodies against caspase-3 and against caspase-cleaved cytokeratin-18. Ca^{2+} chelator (BAPTA-AM) was used to investigate the involvement of calcium in the apoptotic pathway. The involvement of reactive oxygen species was investigated by using the superoxide anion scavenger Tiron [191]. Tiron was also administered to enucleated HCT116 human colon carcinoma cells (cytoblasts) and cisplatin-induced apoptosis was studied. TrxR activity was measured in cell lysates of the colon carcinoma cells after exposition of cisplatin or oxaliplatin.

3.13 Organ of Corti cell cultures (III)

Organs of Corti from P2 or P3 rat cochleae were cultured in well plates and exposed for 24 hours to cisplatin or oxaliplatin. The organs were either used to estimate hair cell loss, or for assessing the enzyme activity of TrxR.

3.14 Thioredoxin reductase activity assay (I and III)

Insulin assay with end-point Trx-dependent insulin reduction [66, 192] was used to measure the activity of TrxR. In paper III, TrxR activity was measured in rat organ of Corti cell cultures and from guinea pig cochlea *in vivo* (organ

of Corti and the lateral wall) [63]. In brief, cells were incubated with recombinant mutant human Trx and insulin, and the absorbance was measured with a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, Ca). In paper I, TrxR activity was measured in cell lysates of human colon carcinoma cells. [193].

3.15 Statistics (I-IV)

For additional information on the statistical calculations, please see the data analysis and statistics sections of each individual paper. The trapezoidal rule was used for the pharmacokinetic evaluations of the AUC studied in paper I. The variability of AUC was determined according to the principles given by Yuan [194]. A two-tailed nonparametric Mann–Whitney U-test was used to compare two independent groups. The wilcoxon rank test was used in the experiments with cultured tumour cells in paper I. Differences for which P-values were 0.05 or less were considered to be statistically significant (papers I, II and IV). A two-way ANOVA was used to compare hair cell densities and, in paper III, one-way ANOVA for the TrxR activity in the *in vitro* studies. One-way ANOVA was also used in paper I, III and IV, when difference in hearing thresholds were calculated, using ABR.

4. RESULTS

4.1 Paper I: Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity

In vitro cell culture studies of apoptosis in HCT116 colon carcinoma cells treated with cisplatin or oxaliplatin

Cisplatin-induced apoptosis of HCT116 colon carcinoma cells was reduced with a calcium chelator (BAPTA-AM) and by superoxide scavenging (Tiron), whereas oxaliplatin-induced apoptosis remained unaffected. These results are shown in Paper I (Fig. 1 B, C). Tiron also reduced apoptosis induced by cisplatin in enucleated HCT116 cells, shown in Paper I (Fig. 1 D).

In vitro cell culture studies on inhibition of TrxR by cisplatin and oxaliplatin in HCT116 colon carcinoma cells

The activity of TrxR was reduced more far after treatment with cisplatin than after oxaliplatin. The results are show in Paper I (Fig. 2).

In vivo studies on hair cell toxicity and total platinum concentration 96 hours after treatment with oxaliplatin or cisplatin

A high dose of cisplatin (12.5 mg/kg) caused a pronounced hair cell loss and total deafness. An equimolar i.v. dose of oxaliplatin (16.6 mg/kg) did not reveal any hair cells loss of and produced only minor changes in the hearing thresholds. The hair cell toxicity is presented in cochleograms in Paper I (Fig. 4). In cochlear tissue, the total platinum concentrations were significantly lower after i.v. administration oxaliplatin (16.6 mg/kg) than after cisplatin (12.5 mg/kg), 96 hours after drug treatment. The results are summarized in Table 1.

Table 1. Hair cells toxicity and total platinum concentration 96 hours after treatment with oxaliplatin or cisplatin

		ABR	Hair cells	Platinum
Oxaliplatin 16.6 mg/kg	n=5	normal hearing	no loss of hair cells	0.12 µg/kg
Cisplatin 12.5 mg/kg	n=5	total deafness	pronounced hair cell loss	0.63 µg/kg
Cisplatin 5 mg/kg	n=5	normal hearing	no loss of hair cells	

In vivo studies of the distribution of cisplatin and oxaliplatin in the cochlea

The AUC values in ST perilymph after i.v. administration of a high dose oxaliplatin (16.6 mg/kg) and a non-ototoxic low dose of cisplatin (5 mg/kg) were similar. AUC for cisplatin 12.5 mg/kg (recalculated from previous data) was significantly higher than AUC for an equimolar dose of oxaliplatin 16.6 mg/kg. The AUC value, the maximal concentration and the half-life of the parent drug in ST perilymph are summarized in table 2 and the pharmacokinetics results are shown in Paper I (Figs. 5, 6 and 7).

Table 2. Pharmacokinetics of cisplatin and oxaliplatin in ST perilymph

		AUC	C max	T 1/2
Oxaliplatin 16.6 mg/kg	n=13	238 µM	4.57 µM (15 min)	12 min
Cisplatin 5 mg/kg	n=10	202 µM	2.95 µM (30 min)	33 min
Cisplatin 12.5 mg/kg	n=21	515 µM (recalculated from previus data)		

Immunohistochemical studies

In non-treated guinea pigs, immunoreactivity of TrxR was expressed in the organ of Corti (inner and outer hair cells), in the lateral wall (stria vascularis and fibrocytes of the spiral ligament), and in the neurons of spiral ganglion. The results are shown in Paper I (Fig. 3).

In summary

The Trx system appear to be involved in cisplatin-induced apoptosis in tumour cells due to a reduced activity of TrxR. Positive immunoreactivity to TrxR in the cochlea indirectly supports the hypothesis that TrxR may be a target and might be involved in the ototoxic effect of cisplatin. A high concentration of cisplatin is present in ST perilymph, whereas the uptake of oxaliplatin is limited. This pharmacokinetic difference seems to be the main explanations for the more pronounced ototoxic side effect from cisplatin as compared to oxaliplatin.

4.2 Paper II: Cochlear pharmacokinetics of cisplatin: An *in vivo* study in the guinea pig

In vivo pharmacokinetics 10–30 min after cisplatin administration

The highest concentrations of cisplatin in blood-UF and ST perilymph sampled from the base of the cochlea were measured on the first sampling occasion, i.e. 10 min after cisplatin administration. The concentration of cisplatin was significantly lower in CSF than in ST perilymph aspirated from the base of the cochlea at target times 10–30 min. The concentrations of cisplatin in the cochlear apex (mean of first sequential samples) were significantly lower

in apex than in samples aspirated from the base of the cochlea at target times 10 and 20 min. The concentration of cisplatin in apex increased significantly after only 20 min. No difference in cisplatin concentration in the cochlear base and apex could be observed at 30 min. These results are shown in Paper II (Fig. 1 and Table 1).

When cisplatin concentrations were calculated from capillaries 1-10, aspirated from the cochlear apex, a significant difference in concentration was found between sample no. 1 and the mean of samples 4 and 5 at 10 min. This gradient was absent at 20 and 30 min. The last sequential samples, 8–10 showed an equivalent concentration of cisplatin, as in CSF at 10 and 20 minutes. The concentration of cisplatin in samples from cochlea base was compared with the mean level of cisplatin in sequential samples 4 and 5 aspirated from the cochlear apex and ST perilymph aspirated directly from the basal turn. A significant difference in concentration was observed at 10 and 20 min, but no difference was seen at 30 minutes. These results are shown in Paper II (Fig. 2 and Table 1).

In vivo pharmacokinetics 30–120 minutes after cisplatin administration

The elimination of cisplatin from ST perilymph aspirated from the base of the cochlea was delayed compared to the blood compartment. At 60 min, the levels of cisplatin in ST perilymph and blood-UF were equivalent. The perilymph–blood ratio then increased with time. The concentration of cisplatin in CSF was significantly lower than in ST perilymph at all target times. ST perilymph sampled from the basal turn of cochlea was analysed for MHC concentration. All samples had a concentration of MHC below the level of detection. The results are shown in Paper II (Figs. 3 and 4).

In summary

Ten minutes after i.v. administration, the concentration of cisplatin in ST perilymph was 4-fold higher in the basal turn of the cochlea compared with the apex. There seems to be an initially greater uptake of cisplatin in the basal part. Elimination of cisplatin from ST perilymph was delayed too.

4.3 Paper III: Cisplatin and oxaliplatin are toxic to outer hair cells and both target thioredoxin reductase in organ of Corti cultures

In vitro studies on organ of Corti cell cultures

Cisplatin and oxaliplatin both reduced the outer hair cell density in cultured organs of Corti. This effect was pronounced in the first half of the basal turn. The results are shown in Paper III (Fig. 1A, B). Exposure of rat organ of Corti cultures for 24 h to cisplatin, or oxaliplatin, reduced TrxR activity significantly. In lateral wall extracts, TrxR activity decreased following exposure to cisplatin and oxaliplatin, respectively (Fig. 3A, B).

Immunohistochemical studies

Positive immunoreactivity to TrxR1 was identified in un-treated P2 rat cochlea. Immunoreactivity to TrxR1 was evident in stria vascularis, organ of Corti, and the spiral ganglion. Positive staining for the enzyme was seen in both inner and outer hair cells, as well as in parts of the developing spiral limbus. Stria vascularis exhibited intense staining. The results are shown in Paper III (Fig. 2).

Measuring TrxR activity in vivo

After i.v. administration of cisplatin and oxaliplatin to guinea pigs no reduction of the enzyme activity of TrxR in the organ of Corti could be verified after 24 hours. The results are shown in Paper III (Fig. 4).

In summary

Both cisplatin and oxaliplatin are toxic to outer hair cells when organs of Corti are directly exposed. TrxR becomes inhibited in parallel with signs of toxicity. TrxR may be a mediator of cisplatin ototoxicity, but in the *in vivo* study TrxR was unaffected.

4.4 Paper IV: Immunohistochemical localization of OCT2 in the cochlea of various species

Immunohistochemical studies

Strong immunoreactivity to OCT2 was localized in the SCs of organ of Corti and in type I SGCs. Similar immunolabelling for OCT2 was identified in rat, guinea pig, and domestic pig cochlea. Positive staining for OCT2 was seen in Deiters´ cells, Hensen´s cells, outer sulcus (Claudius´) and inner sulcus cells, outer and inner pillar cells and in the tympanic covering layer (TCL) localized under the basilar membrane. Type I SGCs displayed intense cytoplasmic OCT2 staining. Two-colour immunofluorescence technique was used on the guinea pig and pig cochlear slides to identify IHCs and OHCs. Positive cytoplasmic and nuclear immunoreactivity to parvalbumin was observed in IHCs with the connecting axons. Weak staining was also seen in the three OHC rows. Double staining with parvalbumin confirmed that no detected immunoreactivity to OCT2 was detected in the IHCs or in the three rows of OHCs. Type I SGCs showed both nuclear and cytoplasmic immunoreactivity to parvalbumin. The results are shown in Paper IV (Figs. 1 and 2).

In vivo study with phenformin

In general there was an obvious individual variability in the ototoxic side effect after cisplatin treatment in both groups. No differences in electrophysiological hearing threshold shifts were observed between the two groups at the three frequencies measured. Morphological evaluation revealed a pronounced loss of OHCs whereas the IHCs were preserved in both groups. No difference in OHC loss was detected between the two groups. The content of platinum in cochlear tissue did not differ between the groups. The results are shown in Paper IV (Table 1).

In summary

OCT2 was localized in the SCs and in type I SGCs in cochleae from three animal species. The findings were verified with immunofluorescence double staining and analysed with confocal microscopy. In the *in vivo* study, phenformin did not reduce the otototoxic side effect of cisplatin.

5. DISCUSSION

5.1 Experimental research

Studies on animals are needed for inner ear research and the guinea pig is a well-established experimental model. The anatomy of the cochlea and hearing in the guinea pig is in many ways similar to that humans but there are many differences between the guinea pig and the human ear [195, 196]. Why just cisplatin causes lesions in the inner ear is still not known, but animals used in this work have improved our knowledge of cisplatin-induced hearing loss. Due to the individual variability in ototoxic effects between animals large groups of animals are needed to achieve statistical significance. However, it is an ethical principle in experimental research to minimize the number of animals.

5.2 DNA-independent apoptosis

Our results support previous studies that cisplatin increases the cell concentration of ROS in cancer cells [197-199] and in cultured hair cells and [68, 200-204], thereby exerting oxidative stress. Due to the differences in therapeutic effects and side effects of cisplatin and oxaliplatin, the difference in the DNA-independent mechanisms of apoptosis were studied [197]. Human colon cancer cells led to similar apoptosis following exposure to cisplatin and oxaliplatin, although via different pathways. The superoxide anion scavenger Tiron reduced apoptosis when treated with cisplatin, but not with oxaliplatin. These findings support the theory of involvement of ROS in the apoptotic effects of cisplatin. The calcium chelator BAPTA-AM reduced apoptosis when treated with cisplatin, whereas oxaliplatin-induced apoptosis was only marginally reduced. This finding also supports the theory that of the involvement of calcium in the apoptotic effects of cisplatin [6].

5.3 Thioredoxin system in the ear

A reduced activity of TrxR following cisplatin treatment could in theory be due either to a reduced amount of the enzyme subsequent to protein degradation, or more likely as intracellular formation of inhibited TrxR protein species [61, 65]. Such inhibited TrxR activity diminishes the capacity of all

Trx-dependent antioxidant defence mechanisms, thereby inducing cell apoptosis. In this work the enzyme activity of TrxR was measured both *in vivo* and *in vitro*.

In paper I reduced TrxR activity could be measured in cisplatin-induced apotosis of colon carcinoma cells. In paper III it was demonstrated for the first time that cisplatin induced an inhibition of TrxR activity in the inner ear tissue, i.e. in organ of Corti culture. In the *in vivo* study, when cisplatin and oxaliplatin were administered i.v., no detectable effect of the overall TrxR activity could be verified after 24 hours, thus leaving numerous questions unanswered regarding the role of the Trx system in cisplatin ototoxicity. Nevertheless, this finding does not exclude an effect of cisplatin on TrxR activity *in vivo*. There could be several reasons for the failure to demonstrate TrxR inhibition in the *in vivo* study. It is known that an ototoxic effect of cisplatin becomes evident 96 hours after i.v. administration of cisplatin 8 mg/kg [79, 105].

In this study the activity of TrxR was evaluated after only 24 hours. One might object that the time window for measuring TrxR activity was inappropriate. One obvious difference between the *in vivo* and the *in vitro* study of TrxR activity was the exposed concentration of cisplatin that might have affected the inhibition of TrxR. Data from paper II show that a maximum concentration of cisplatin in ST perilymph was 5.86 ± 1.43 μM and cisplatin could be measured up to 120 min after cisplatin (8 mg/kg) was administered i.v. In the *in vitro* study, organ of Corti cell cultures were exposed to 20 μM cisplatin for 24 hours. Positive immunohistochemical staining of TrxR in guinea pig cochlea (paper I) and in rat cochlea (paper III) supports the theory that cisplatin induced apoptosis of OHCs might involve an inhibition of TrxR activity, although it could not be demonstrated *in vivo*.

5.4 The importance of inner ear pharmacokinetics for cisplatin ototoxicity

Little has earlier been known about cisplatin transport to the hair cells or about the inner ear pharmacokinetics of cisplatin. With the present studies we have brought new insights into inner ear pharmacokinetics, showing that the passage of cisplatin across blood–perilymph barrier and the pharmacokinetics of cisplatin in the cochlea is important for the ototoxic effect of the drug. *In vivo* experiments are of importance for ototoxic studies, compared with studies based only on cell cultures when the inner ear barriers are circumven-

ted. However, the concentration of cisplatin in ST perilymph may be only a rough estimate of the accessible drug concentration in the cochlea and there is no simple relationship between ST perilymph concentration and pharmacological effect expressed as a loss of OHCs. Laurell and co-workers (1995) showed that cisplatin could be measured in ST perilymph after i.v. injection of cisplatin (12.5 mg/kg). Ekborn and co-workers [79, 121, 125] performed pharmacokinetic studies on cisplatin and MHC in blood.

In paper I it was shown that the lack of an ototoxic effect of oxaliplatin was due to a limited passage to ST perilymph. These findings were strongly supported by the results published in paper III showing that direct administration of oxaliplatin to the hair cells *in vitro* caused hair cell loss. In paper II it was demonstrated that the level of cisplatin in the cochlear base was more than 4-fould than in the cochlear apex, 10 min after cisplatin administration. It can be speculated if the initially high concentration of cisplatin in the base might favor the toxic effect of cisplatin on OHCs, thereby inducing a longitudinal pattern of injury along the cochlear duct. Delayed elimination of cisplatin from ST perilymph compared from blood is shown in paper II. This finding suggested a prolonged exposure of the organ of Corti to cisplatin, which could be an important factor for the cytotoxic effect in the cochlea [80].

When perilymph is sampled from the base of the cochlea there is always a high risk of CSF contamination through the cochlear aqueduct. Salt et al. showed a contamination of approx. 15% CSF when 1 µl ST perilymph was aspirated from the basal turn of the cochlea [181, 205]. In our studies the concentrations of cisplatin in CSF were lower than in ST perilymph indicating that the estimated concentration of cisplatin could never be impossibly high. Cisplatin was also clearly shown to penetrate the blood–CSF barrier, due to the measurement of cisplatin in CSF in papers I and II [80].

In paper II a gradient of cisplatin concentration in ST could was established due to the sequential sampling technique from the cochlear apex [182]. The fourth and fifth samples, of one µL, are regarded as representative of ST perilymph from the basal turn and the last samples, no. 8–10 to be highly contaminated with CSF [181, 205]. The results of this study support those above showing a concentration gradient in the cochlea with an initially higher concentration of cisplatin in the base compared to apex. At 10 min there was a significant difference in concentration between sample no. 1 and the mean of samples no. 4 and 5.

Ekborn and co-workers (2003) showed that i.v. administration of MHC was more ototoxic than cisplatin [79]. In paper II, MHC was analysed in ST perilymph after i.v. cisplatin, but the concentration was below the limit of detection [121, 125], not unexpected as the concentration of MHC in blood is only 8–9% of the total amount of cisplatin in blood [77, 121]. However, one cannot deny that MHC may be involved in the ototoxic effect in the inner ear fluids, as supported by the studies of Ekborn and co-workers (2003) [79].

The blood concentration of cisplatin is crucial for the inner ear pharmacokinetics of cisplatin, as demonstrated by the results in papers I and II. A low variability of cisplatin in blood–UF was demonstrated and this is also known from previous studies in both experimental animals and in patients [4, 79, 121, 125, 206]. When an i.v. low dose of cisplatin (5 mg/kg) was administered the maximum concentration (2.95 μM) in ST perilymph was found after 33 min. After an ototoxic dose of cisplatin (8 mg/kg) the maximum concentration (5.86 μM) in ST perilymph was observed after only 10 min. Such differences in peak time in ST perilymph were rather surprising, possibly showing that the transport of cisplatin to the inner ear is not directly proportional to its concentration in blood.

5.5 Transport proteins and cisplatin ototoxicity

Drug transport is a widening scientific field of relevance for cisplatin cytotoxicity and its side effects. It has been demonstrated that profuse expression of Ctrl in the tumour is associated with a reduced antineoplastic effect of cisplatin in patients with ovarian cancer. This was attributed to an enhanced efflux of cisplatin from ovarian cancer cells which reduces the therapeutic response [207]. Due to similarities between the nephrotoxic and ototoxic effects of cisplatin, an active transport mechanism has been suggested for the inner ear and the kidney. Recent studies have demonstrated that OCT2 is involved in cisplatin-induced oto- and nephrotoxicity [39]. OCT2 is present in the cell membrane of the renal tubular cells and is known to be a specific transport protein for the uptake and accumulation of cisplatin, resulting in a nephrotoxic side effect [208, 209].

OCT2 was earlier immunohistochemically localized in the mouse cochlea [39, 168]. In cisplatin-treated OCT2 knockout mice, there were no signs of ototoxicity and only mild nephrotoxicity [39]. These studies were actually published after our immunohistochemical finding of the localization of OCT2 in the cochlea. In paper IV, OCT2 was identified immunohisochemically in

the cochlea. Thanks to double staining with parvalbumin, OCT2 could be clearly localized in the SC of the organ of Corti and in the type I spiral SGCs.

These findings were surprising due to the assumed localization of OCT2 in stria vascularis, with active passage through the intrastrial fluid–blood barrier. Our findings support previous studies where cisplatin reportedly to induce toxic lesions in the SCs in the organ of Corti, mainly in Deiters´ cells [104, 105]. Several ion channels [210] and gap junction proteins such as connexins are located in the SCs [187]. They are probably involved in the transport of potassium that is released by the hair cells and re-cycled to the endolymph compartment [103]. A reduced function of the SCs would probably induce secondary apoptosis of the hair cells [211]. The concentration of cisplatin in ST perilymph would therefore be of a considerable importance for these toxic effects but it is not known how cisplatin enters the OHCs [212, 213].

5.6 Blood–perilymph barrier and cisplatin ototoxicity

The finding that cisplatin can be measured in ST perilymph, at low concentrations after a low i.v. dose of (5 mg/kg), demonstrates its ability to penetrate the blood–perilymph barrier [80] i.e. vessels in the spiral portion of the cochlea [144]. The concept of active transport through the blood–perilymph and the intrastrial fluid–blood barrier could not be documented. The findings in the present work more support a passive transport to the different inner ear compartment and an active uptake to the target cells for cisplatin ototoxicity, in the organ of Corti involving interaction between the SCs and OHCs.

5.7 Individual variability of ototoxicity

The pathophysiology behind the individual variability for cisplatin-induced hearing loss is not known and was not studied in this work but some parameters elucidated can be surmised to be involved. One can speculate that there might be an individual variation in redox system activity in the cochlea due to variations in the GSH and Trx redox systems. This speculation might be supported by Oldenburg and co-workers' findings (2007) showing a specific genotype of the enzyme glutathione S-transferase explaning differences in cisplatin-induced ototoxicity between individuals [214]. Another reason could for the individual susceptibility to the drug could be variations in active transport mechanisms for cisplatin to the target cells for ototoxic effect. There could also be an individual passage of cisplatin to ST perilymph, i.e. across the blood–perilymph barrier due to variations in the microvascular anatomy.

5.8 Apoptosis of OHCs in the base of the cochlea

It is well known is that the basal parts of the cochlea are most seriously affected by cisplatin [122-124, 215, 216]. Why there is a difference in OHC loss along the length of the cochlea is not understood. According to the present results there is an early high distribution of cisplatin to the cochlear base that could initiate an increased cytotoxic activity in the vulnerable cells for hearing. There might also be a variation in apoptotic mechanisms along the cochlea [217, 218]. The GSH and Trx systems might be less active in the cochlear base [70]. There could also be a variation in specific cisplatin-transport proteins along the cochlea [80, 206].

5.9 Protection from cisplatin ototoxicity

Although extensive research has been performed to protect hearing during cisplatin treatment, no clinical oto-protective therapy has yet succeeded. The best treatment would be to protect the hearing and the end organs without interfering with the antitumour effect of cisplatin. Studies on cisplatin kinetics *in vivo* will help to shed light on possible ways to prevent ototoxicity. There are a few findings in this work that might well be of interest for the development of a protective approach against the ototoxic side effects of cisplatin.

One theoretical approach for oto-protection would be to block any active transport mechanism of cisplatin to the sensory hearing cells, as was studied in paper IV. Phenoformin, an anti-diabetic drug of the biguanide class was administered as a competetiv antagonsist to cisplatin for the binding to OCT2 [219, 220]. In clinic phenformin is withdrawn from the market due to its development of lactic acidosis. Phenformin was administered i.v. to guinea pig and the ototoxic effect of cisplatin was evaluated, no protection could be seen. Due to our immunohistological finding that OCT2 was localized in the deepest compartment of the inner ear, in the supporting cells, OCT2 can be hypothesized as being involved in a more direct uptake of cisplatin by the hair cells. In previous studies, cimetidine [39, 178, 179, 221] and copper sulphate [38, 168] were shown to prevent cisplatin-induced ototoxicity by interfering with a proposed active transport mechanism. When transport inhibitors or competitors are given systemically, they interact with the parent drug and local administration is therefore to be preferred [180]. Further studies are needed to evaluate the passage of cisplatin from blood to the fluid-filled compartments of the cochlea.

From the pharmacokinetic results one can point out that very low concentrations of cisplatin in ST perilymph are found when a non-ototoxic dose of cisplatin is administered i.v. A treatment that would reduce an elevated concentration of cisplatin in ST perilymph, and then primarily in the basal parts would thereby have a potential oto-protective effect.

5.10 Limitations of the studies

Due to our knowledge that the most apical parts of the hair cells are situated in SM endolymph, the concentration of cisplatin in this compartment would have been of interest to analyse as one part of the pharmacokinetic studies. To sample endolymph from SM is a complicated procedure that could not be performed in our animal model. In paper III there were two different species in the *in vitro* and *in vivo* study, which may have affected the outcome. In paper IV it would have been of interest to assess the concentration of phenformin in ST perilymph. Most important of all, in the *in vivo* studies, larger study groups would have been an advantage due to the wide ototoxic variations after treatment with cisplatin. For ethical reasons, however, this would have been difficult to perform.

5.11 Clinical applications and future studies

Apart from drug-induced hearing loss, loss of hearing in the high frequencies also follows noise trauma and age-related hearing loss. The OHCs in the cochlear base seem to be the most sensitive, in general. If studies using ototoxic drugs can improve our knowledge of the apoptotic mechanisms of the OHCs, this might also have implications for age-related hearing loss and noise-induced hearing loss. Further study of a proposed active transport mechanism of cisplatin to the inner ear and more knowledge of the vascular microanatomy of the inner ear barriers are also of importance in the development of cisplatin oto-protection. The importance of TrxR as a molecular target in cisplatin ototoxicity calls for further study. The individual ototoxic effects of cisplatin also need to be better studied. Once again, conclusions from ototoxic studies based only on cell cultures when the inner ear barriers are circumvented may be a little value unless they are combined with experiments using a reliable *in vivo* model.

6. CONCLUSIONS

The general conclusions of the work presented in this thesis can be summarized as follows:

The *in vivo* pharmacokinetics studies showed an extent transport of cisplatin across the blood–perilymph barrier and only a limited passage of oxaliplatin to ST perilymph. These pharmacokinetic findings appear to form one of the main explanations for the differences in ototoxic side effects between cisplatin and oxaliplatin. The initial high concentration of cisplatin in the base of the cochlea and the delayed elimination of cisplatin from ST perilymph may correlate to the loss of OHCs in this region of the cochlea. Administration of phenformin did not provide any oto-protection.

The *in vitro* studies showed an equal loss of hair cells in organ of Corti cell cultures following direct exposure of cisplatin or oxaliplatin in the growth medium. This support the hypothesis that the blood–perilymph barrier is of fundamental importance for the ototoxic effects of cisplatin. The Trx redox system seems to be involved in cisplatin-induced ototoxicity. Activity of TrxR was reduced in relation to apoptosis of the hair cells after direct administration of cisplatin to organ of Corti cultures. Cisplatin-induced apotosis in human colon carcinoma cells also reduced the activity of TrxR.

In the immunohistochemical studies, TrxR and OCT2 were identified in the cochlea. Positive immunoreactivity to TrxR in indirectly supports the hypothesis that TrxR targeting, at least in part, contributes to cisplatin-induced apoptosis of the vulnerable cells of hearing. OCT2 was identified in SCs and type I SGCs, indicating that OCT2 is primarily not involved in the uptake of cisplatin from the systemic circulation, but rather from deeper compartments of the cochlea.

7. POPULÄRVETENSKAPLIG SAMMANFATTNING

Det platina-innehållande cytostatikapreparatet cisplatin har ototoxiska biverkningar. Den irreversibla sensorineurala hörselnedsättningen resulterar ofta i att cisplatin exkluderas och därmed kan effekten av cytostatikabehandlingen påverkas. Det övergripade målet med avhandlingsarbetet var att få mer kunskap om cisplatin-orsakad hörselnedsättning. Cisplatins farmakokinetik har analyserats i innerörats vätska. DNA-oberoende mekanismer som kan vara involverade i cisplatins apototiska effekt på innerörats cellförband har studerats. Med immunohistokemi har ett potentiellt transportprotein för cisplatin identifierats i innerörat. Resultaten från avhandlingens arbeten ger en ökad kunskap om mekanismer bakom cisplatin-orsakad hörselnedsättning. Detta kan bidra till en framtida utveckling av protektiva åtgärder i syfte att skydda cisplatin-behandlade patienter från att drabbas av hörselnedsättning. Nedan följer en sammanfattning av artiklarna I-IV i avhandlingsarbetet:

I: Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity. *"En studie på cisplatins och oxaliplatins toxiska effekter och betydelsen av cisplatins farmakokinetik i koklean för dess ototoxicitet"*.

Resultaten i artikel I visade att cisplatins farmakokinetik har betydelse för preparatets ototoxicitet. Höga nivåer cisplatin uppmättes i i scala tympani perilymfa efter att cisplatin administrerats i.v. till marsvin. Resultaten jämfördes med data från motsvarande försök med oxaliplatin, som saknar ototoxisk biverkan. Endast låga halter oxaliplatin kunde analyseras i scala tympani perilymfa och detta skulle kunna förkara avsaknad av ototoxisk biverkan. Vid cisplatin-inducerad apoptos av odlade colon cancer celler kunde man påvisa en minskad aktivitet av enzymet thioredoxin reduktas. Positiv immunoreaktivitet för thioredoxin reduktas kunde identifieras i koklean hos marsvin. Dessa fynd talar för att thioredoxin systemet kan vara involverat i DNA-oberoende apoptos av innerörats hårceller.

II: Cochlear pharmaockinetics of cisplatin – an in vivo study in the guinea pig. *"En in vivo studie av cisplatins farmakokinetik i koklean hos marsvin"*.

I artikel II har cisplatins farmakokinetik studerats i scala tympani perilymfa hos marsvin. Resultaten visar en initialt hög koncentration av cisplatin i scala

tympani perilymfa i kokleans bas 10 min efter i.v. administration av cisplatin till marsvin. 4 ggr högre koncentration uppmättes i kokleans bas jämfört med apex. Detta kan vara en del av orsaken varför det är hårceller i kokleans bas som i första hand skadas av cisplatin. Cisplatin hade även en långsammare elimination från perilymfan jämfört med från blodet.

III: Cisplatin and Oxaliplatin are toxic to cochlear outer hair cells and both target thioredoxin reductase in organ of Corti cultures. *"En studie som visar att en direkt exponering av oxaliplatin till organ of Corti cell kultur är toxiskt för kokleans yttre hårceller och ger en minskad aktivitet av thioredoxin reduktas".*

Artikel III utvärderar den toxiska effekten på hårceller efter en direkt exposition av cisplatin och oxaliplatin till odlingsmedlet i cellkultur från organ of Corti. Thioredoxin systemet har studerats i relation till DNA-oberoende apoptos av hårceller både *in vivo* och *in vitro*. Resultaten visar att cisplatin och oxaliplatin har likartad toxisk effekt på de yttre hårcellerna vid en direkt exponering i organkultur och att thioredoxin systemet är involverat i de apoptotiska mekanismerna. Nivån av enzymet thioredoxin reduktas i innerörats cellförband påverkas dock inte efter administrering av cisplatin och oxaliplatin *in vivo*. Resultaten stärker fynden från arbete I, det vill säga att transporten av cisplatin till innerörat är av betydelse för dess ototoxicitet.

IV: Immunohistochemical localization of OCT2 in the cochlea of various species. *"En studie som med immunohistokemi lokaliserar OCT2 i koklean hos olika arter".*

Artikel IV undersöker förekomsten och lokalisationen i innerörat av ett transport protein, som kan vara involverat i cisplatins ototoxiska effekt. Positiv immunoreaktivitet för organisk katjontransportör 2 (OCT2) kunde identifieras i det kortiska organets stödjeceller och i spriral ganglion celler hos marsvin, råtta och gris. Lokalisationen av OCT2 talar för att transportproteinet inte medverkar i transporten av cisplatin från blodbanan men indikerar att det kan vara delaktig i en transport via stödjecellerna till yttre hårcellerna. Phenformin administrerades i.v. som en blockerare till OCT2. I aktuell dos gav inte phenformin något skydd mot cisplatin-utlöst ototoxicitet.

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